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Research Paper

Enhancement of activity in the Cancer immune system due to the presence of microcomponents when Exposed to Photodynamic: An *in Vitro* Experiment

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ABSTRACT

The application of photodynamic therapy (PDT) has aimed at destroying tumor cells and microbiological control in infections. Experimentation *in vitro* with individual elements of a biological system enables the study of mechanism of action and provides directions to *in vivo* applications where cancer coexists with normal cells and microorganisms, whether opportunistic or not. The action of the combined set of elements leads to a different behavior from each of the components isolated. In this study, we created a simple model to investigate the effects of PDT (1) on the coculture of macrophages (2) and melanoma cells (3) infected with *E. coli* (4) under different combinations. Comparing the 4 individual elements of the system interacting with each other to all of them interacting together, we found that macrophage phagocytosis depends on the order in which the elements are exposed to PDT. That indicates the behavior of macrophages depends on their relationship with the microenvironment. Applying the photodynamic effect to each component separately or to all of them together results in different outcomes. These *in vitro* experiments provide pathways to understand or to design new and more efficient applications of PDT.

1. Introduction

Cancer is a leading cause of death worldwide, responsible for almost 10 million deaths (one in six deaths) [1,2]. Advanced and fast-progressing cancers are especially difficult to treat since they are either insensitive or quickly develop a resistance to treatment. One of the proposed mechanisms of overcoming resistance involves a change in tumor microenvironment on the tissue level (intratumor cell interactions with the immune system) and on the cellular level (reactive oxygen species production, DNA damage, membrane permeabilization, and other reactions). If the patient has a weakened immune system, the introduction of a disabled pathogen or its fragments can reactivate its function. Other changes in the tumor microenvironment include cancer development altered by bacteria by shifting metabolism through dysbiosis [3] and the utilization of bacteria in cancer therapeutics [4,5].

In recent years, immunotherapy has emerged as a new strategy for cancer treatment. The evolution of immunotherapy, however, took over 100 years. Bacteria-based cancer immunotherapy was first attempted in the 19th century by William Coley [6] who used live or heat-inactivated *Streptococcus pyogenes* and *Serratia marcescens* to treat patients with inoperable cancer. This strategy resulted in an increase of over 10 years in the life span of 30% of patients [6]. Continuing this promising strategy may involve whole naïve bacteria, inactivated by heat, sonication, UV irradiation, or the use of bioengineered versions of those bacteria lacking pathogenic genes [7]. Also, the subproducts or components stimulate the immune system to eradicate the bacteria together with tumor cells and reverse the immunosuppressive microenvironment [8,11]. Unlike other therapies, the effectiveness of tumor-targeting bacteria is not related to the type of cancer. When infection is initiated within the tumor, it is followed by unmodified antitumor immune responses. Therapeutic approaches using live tumor-targeting bacteria can either be applied as monotherapy or combined with other anticancer

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therapies to achieve better effectiveness [8,11,13].

In this work, we illustrate how photodynamic therapy increases its action in an *in vitro* cancer model when applied together with *E. coli* infection by initiating melanoma regression and activating macrophages to increase phagocytosis.

We used a simple model of coculture of macrophages and melanoma and introduced bacterial infection with *E. coli*, followed by PDT with different photosensitizer (PS) concentrations or effective light doses, to observe possibly enhanced therapeutic benefits against cancer. The most profound enhancement of anticancer activity was observed when immune cells were treated with PDT during infection with E. coli. Because initiates an immune response even in the cancer immunosuppressive microenvironment, the pathogen can enhance the specific immune recognition and elimination of cancer cells by macrophages [5,9,10,11].

Because macrophages often uptake more photosensitizer (PS) than melanoma, by bringing them physically close to each other makes the PDT more effective. Addition of bacteria further serves to signal macrophages to follow the site of infection whether the infection is naturally occurring in the hypoxic necrotizing part of the cancer or artificially introduced as part of therapy [10,12]. The observations of this present study provide a starting point for a series of experiments involving addition of extra components in the cancer microenvironment as well as investigating their possible interactions to optimize the final results.

2. Materials and methods

Cell lines: J774A.1 cell line it is a BALB/c monocyte macrophage cell line isolated in 1968 from the ascites of an adult, female mouse with reticulum cell sarcoma (ATCC TIB-67 ™). It was purchased from ATCC and cultured in (75cm2) VWR Tissue Culture Flask in DMEM nutrient mixture supplemented with Glucose and Glutamine, 10% fetal bovine serum (FBS) and maintained according to ATCC protocol. The day before the experiment 96 wells plates were seeded with 1×10^6 cells and grown overnight.

B16-F10 cell line is an adherent skin cell line with a morphology of spindle-shaped and epithelial-like cells, originally isolated from mice with melanoma (B16-F10 (ATCC CRL-6475). It was purchased from ATCC and cultured the same as **J774A.1** macrophages (described before). After 19 h of incubation at 37 °C, the melanoma cells in 96 well plate was rinsed with Dulbecco's Phosphate Buffered Saline 1x (PBS) sterile-Filtered (ATCC®) followed by an incubation with PBS and glucose (10mM) (Sigma-Aldrich®) and photosensitizer solutions for 30 mins in dark at 37 C.

Coculture of macrophages with melanoma was seeded at the same final concentration (1×10^6) and same time before the experiment as single line cultures described above, but containing 50% of each cell lines growing together.

2.1. Escherichia coli

The *E. coli* (ATCC25922 and ATCC25922GFP) was purchased from American Type Culture Collection. The day before the experiment, a pre-inoculum solution was prepared adding a proportion of 1:9 of bacteria from cryosample and Brain heart infusion (BHI) media sample was incubated in the incubator for 16 h and rotating for 150 rpm at 37°C. In sequence, bacteria cells were washed twice using Phosphate Buffer Solution (PBS), the inoculum was standardized to a 0.3 OD_{600} of initial concentration, corresponding to 1.10^7 CFU/mL in a 10 ml solution.

Infections experiments were performed as previously described by Qin QM et al [14]. Eukaryotic ells were plated to 96 wells at 2.5×10^6 and bacterial suspension was 10^6 CFU/ml. In some experiments we treated *E. coli* with PDT and then added to the coculture of macrophages and melanoma, in others we used bacteria not treated with PDT. Also, we tested both the infection of the cell culture after PDT and the coculture infected first, followed by PDT. Regardless of the setting, the studied cells (melanoma, macrophages and E. coli) were incubated with photosensitizer for 30 min in dark at 37 °C. Time of incubation of eukaryotic cells with bacteria was also 30 min. After the bacteria suspension was removed, we used 40ug/ml Gentamycin (Sigma Aldrich) to remove not phagocytosed bacteria from plate.

Photosensitizer – we used Photodithazine® (PDZ, chlorin-e6) as a photosensitizer. Stock solution was prepared at 0.5 mg/ml, working solutions were diluted using PBS. After testing many concentrations, we chose the 1×10^{-4} mg/ml for all the studies.

2.2. Irradiation device

The irradiation was performed by a set of 24 LEDs (developed by the Laboratory of Technological Support (LAT - Institute of Physics of São Carlos, IFSC / USP/Brazil) homogenously emitting light at 660 nm with fluence of 40mW/cm^2 which is calibrated for the illumination of 96 well plates during the cell culture exposure. Each dose was applied according to the dosimeter indicated time of exposure.

2.3. Cell viability determination

Three different assays were applied for analyzing the results: **MTS** 3-(4,5-dimethylthiazol-2-yl)−5-(3-carboxymethoxyphenyl)−2-(4-sulfophenyl)−2H-tetrazolium (CellTiter96 Aqueous Proliferation Assay; Promega Corp.); **LDH** (lactate dehydrogenase) Cytotox 96 kit (Promega Corp., Madison, WI) and **ToxiLight®** (ToxiLight™ Non-Destructive Cytotoxicity BioAssay Kit.

After exposure of melanoma and macrophages cultures without E. coli the aliquots were removed and toxicity in culture was measured by LDH (lactate dehydrogenase) release into the medium assay was used according to the manufacturer's directions and monitored at 492 nm vs 680 nm using the Infinite 200 Pro (Tecan Austria GmbH) plate reader.

The remaining medium was discarded, and the cells were washed with PBS and incubated with PBS/10 mM glucose. The cell viability was then measured using the MTS assay monitored at 492 nm vs 620 nm by the same plate reader.

Toxicity in experiments involving infection of E. coli was evaluated by Lonza™ToxiLight assay, according to the manufacture's direction since LDH assay interferences with bacterial infection and underestimates toxicity [7]. ToxiLight assay measures adenylate kinase (AK), via monitoring luminescence and can detect a dynamic range of over 5 orders of magnitude.

2.4. Imaging flow cytometry phagocytosis quantitative analysis

Macrophages were cultured with FITC-labeled E. coli with or without PDT and phagocytosis was analyzed using an Image Stream X MarkII imaging flow cytometry (Cytek/Amnis), equipped with a 400 mW 488 nm argon-ion laser, and a 60x objective to detect the FITC fluorescence (528/65 nm bandpass; Channel 2). The Cytek Amnis acquistion software, INSPIRE, was used to collect data. The samples were collected on low-speed setting with the 60x objective with the 488 nm laser set at 25 mW of power. An average of more than 2000 singlet counts of each sample were obtained. The images and data were analyzed using IDEAS image analysis software (Cytek/Amnis). The gating strategy for how the cells were analyzed is described in the gating strategy figure and figure legend in the Supplemental Fig. 3. The masks used for image analysis were the Erode (M01,6) mask for internalization of the FITC-labeled *E. coli* and the Intensity (Watershed (Spot (M02, Ch02, Bright, 6, 3, 1)), 3–500, 0.5–1), Ch2, 750–4095) mask was used for spot counting of the FITC-labeled E. coli within the macrophages.

2.5. FTIR

Spectra were collected in the range of 650 to 4500 cm^{-1} infrared region using a FTIR spectrometer (Agilent Cary160) on ATR sampling mode. The crystal was cleaned, a background was measure, and then 10

μL of each sample was collected. The spectra were acquired from untreated macrophages cells infected by *E. coli*, macrophages treated with PDT (1.10^{-4} mg/mL-30J/cm^2) and infected by *E. coli*, macrophages infected with E. coli treated with PDT (0,01 mg/mL-30J/cm^2) and macrophages cells infected by *E. coli*, and both treated with PDT (1.10^{-4} mg/mL-30J/cm^2).

The baseline correction was performed by OriginLab® software for data analyses. Principal Component Analysis (PCA) was applied on preprocessed second derivatives spectra. Biochemical changes were assessed using PCA-loadings and accuracy of classification was obtained from PCA.

2.6. Statistical analysis

The data were presented as means and SD (standard deviation). Two-group comparisons were performed by Student's t-test and using One-way ANOVA followed by Tukey test considering two-tailed p values < 0.05 statistically significant. ANOVA analyses were performed using Excel software. PCA analyses were performed using Octave, a free software.

3. Results and discussion

When conducting any experiment involving photodynamic action, it is first necessary to investigate the optimal conditions for the operation of parameters such as light dosage and concentration of the photosensitizer to be used. The determination of these parameters initially guides the choices for the components involved. In the preliminary studies, the idea was to have doses of light and photosensitizer that would not destructively affect the macrophages so that it would be possible to verify their influence on melanoma cells. On the other hand, the effect must be observable to be sure that photodynamic action occurs, even if it is not very pronounced.

3.1. Macrophages

Fig. 1 shows toxicity under photodynamic action for macrophages J774A.1 subjected to different concentrations of PS and different doses of light.

We started with testing toxicity of 10^{-4} mg/ml and 10^{-3} mg/ml concentrations of PDZ and different light doses on macrophages J774A.1. Since we were interested in the investigation of bacteria possible engagement in cancer photodynamic therapy, we chose the lowest effective parameters of PDT to be able to detect their influence. It was 1 x10^{-4} mg/ml PDZ and 30J/cm^2 of light emitted at 660 nm. In those conditions, we detected that less than 10 % of toxicity on the macrophages compared to the control was observed. Doses 40 J/cm^2 and 50 J/cm^2 caused 20 to 40 % for 10^{-4} mg/ml. Above this concentration the results indicated a much more pronounced toxicity for the cells. Therefore, we decided to choose the 10^{-4} mg/ml PDZ for our experiments to be able to see if combined therapy increases the damage of melanoma. Light doses of 10 and 20J/cm^2 were not changing the PDT effect compared to dark control. Once the concentration of PDZ was selected, the viability of the macrophages was investigated, in different light doses. Fig. 2 shows the results obtained for the viability test at a fixed concentration and for different light doses.

Cell viability is a measure of how healthy the cell remains after undergoing the procedure. The viability observed clearly shows that the chosen conditions guarantee the best possible preservation of the conditions of the macrophages while allowing minimal damage affected by the photodynamic action. The choice of the conditions already mentioned guarantees, in this way, the desired effect, which is the minimum toxicity and the maximum viability.

Experiments demonstrate grater toxicity at higher doses and, as shown in Fig. 2, a compromise in viability above 60 % at higher doses of light. Equivalently, toxicity also increases sharply above 30 J/cm^2. All evidence indicates that macrophages at a given light dose show a low variation in viability for a fixed concentration of photosensitizer, starting a steady decline after a certain value. In real applications, minimizing the destruction of essential cells of the immune system can be a good strategy in selecting of PDT conditions.

Once the ideal conditions for the macrophages have been chosen, the next step is the addition of *E. coli* bacteria to check whether the presence of the bacteria causes effects on both the toxicity and the and viability of eucaryotic cells. The presence of bacteria places macrophages in new state of metabolism. This effort could certainly change the behavior already investigated. The study was carried out in three different situations: (1) performing the PDT on the cell culture and then adding the bacteria, (2) adding the bacteria and then performing the PDT, or finally (3) performing the PDT on the bacteria and then adding them to the cell culture. The overall results are presented in the Fig. 3.

The possible sequential combinations of the three essential elements under evaluation make sense because PDT will act differently in each one, and the temporal sequence in which the facts occur can greatly affect the results of mutual action. In virtually all combinations, significant results of macrophage toxicity or viability modifications were found in relation to the presence of *E. coli*. However, it should be noted, that performing PDT on macrophages before adding *E. coli* resulted in a slight decrease in their viability. This effect was initially expected, since the conditions of PDT application had previously demonstrated small variation in macrophages alone, probably due to structural or functional damage caused by the oxidative action of PDT. In toxicity and viability parameters, we can consider absence or minimal variation with the addition of *E. coli* and PDT.

To complete the observations of macrophage behavior in the

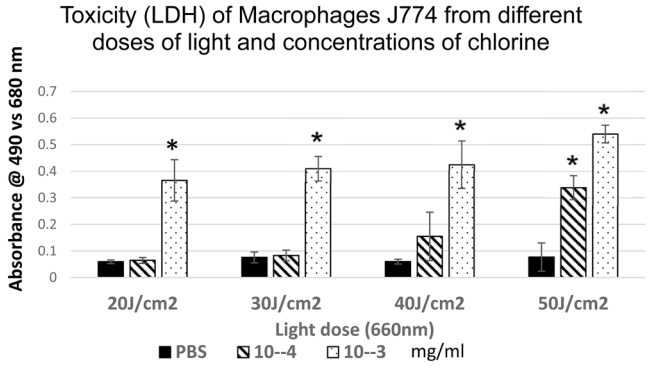

Fig. 1. Evaluation of toxicity of Macrophages J774A.1 with chlorine and light doses at 660 nm. The used concentrations were 10^{-4} mg/ml and 10^{-3} mg/ml. Data were analyzed by two-way ANOVA and symbol * indicates p-value <0.05.

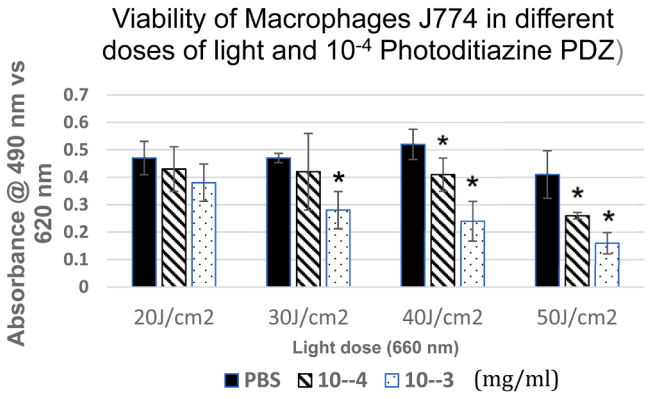

Fig. 2. Viability of Macrophages J774A.1 in different doses of light and concentrations of chlorine. Data were analyzed by two-way ANOVA and symbol * indicates p-value <0.05.

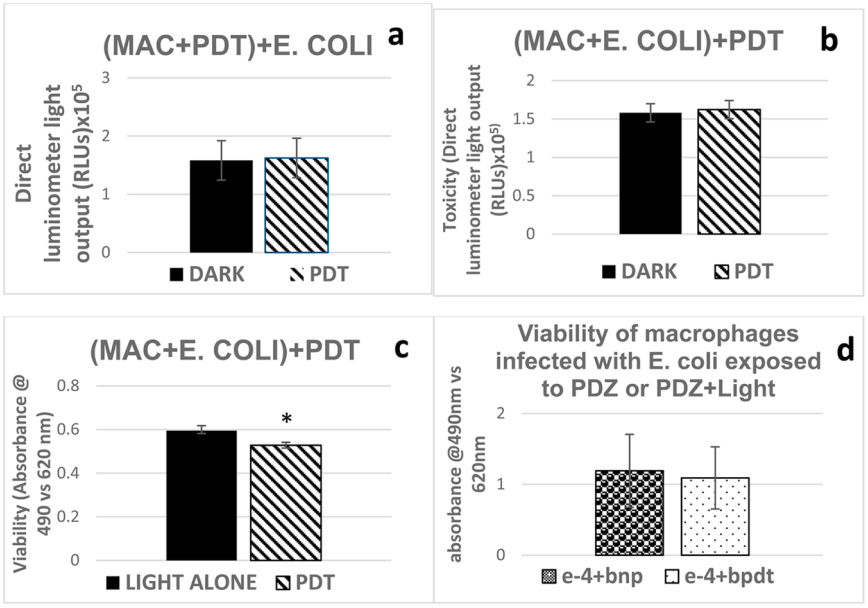

Fig. 3. Toxicity of macrophages J774A.1, exposed to 30J/cm^2 of light in two sequences: (a) First exposure to PDT followed by infection of E. coli. (b) Addition of E. coli infection followed by the PDT. **Viability** of the macrophages in different conditions of infectivity and PDT: (c) Exposure to PDT followed by *E. coli* infection; and (d) application of PDT in the bacteria before addition to the cell culture (e-4+bpdt) vs bacteria incubated in PDZ but not exposed to light (e-4+bnp). Data were analyzed by Student *t*-test and symbol * indicates p-value <0.05.

presence of PDT and bacteria, macrophage activity parameters were monitored using flow cytometry to observe the phagocytosis process of *E. coli* in different situations. The results are presented in Fig. 4. While performing PDT in each component individually decreases phagocytosis, we noted that macrophages show increased phagocytosis of bacteria when both are exposed to of PDT, even though no variations in macrophage viability were observed under these conditions. This result is intrinsically interesting information for the immune system's overall reactions to PDT-treated regions.

The activity of phagocytosis is somewhat diminished when PDT is performed on the *E. coli* prior to exposure to macrophages. The explanation is that the macrophages no longer recognize the bacteria with the same accuracy once it has been modified/destroyed by PDT action. But when both macrophages and bacteria are incubated simultaneously, however, PDT induces the best macrophage phagocytosis action possibly by a mechanism of increasing the liberation of biochemical during the PDT on the bacteria and simultaneously increasing the sensitivity of the macrophages under oxidative stress.

Generally speaking, disrupting macrophage function, even below the measurable viability threshold, has an impact on phagocytosis. When bacteria, macrophages, and PDT are present together, the effect of phagocytosis is amplified; yet, when one of these organisms is exposed to PDT alone, phagocytosis is reduced. This implies that PDT is destroying the macrophages' bacterial recognition capability. This also could be because the bacteria have likely taken up a large amount of PDZ and the PDT treatment created a lot of intracellular reactive oxygen species (ROS) in the bacteria. This likely caused bacterial cell death and compromised membranes therefore leaking out ROS. These ROS could have had a negative effect on the membrane of the macrophage resulting in less phagocytosis of bacteria [15]. These above are only hypotheses that remain to be further investigated.

Also the decrease in phagocytosis of the MAC PDT treated only having less phagocytosis could be from increased ROS production in the macrophage affecting cytoskeletal components induced during phagocytosis [16].

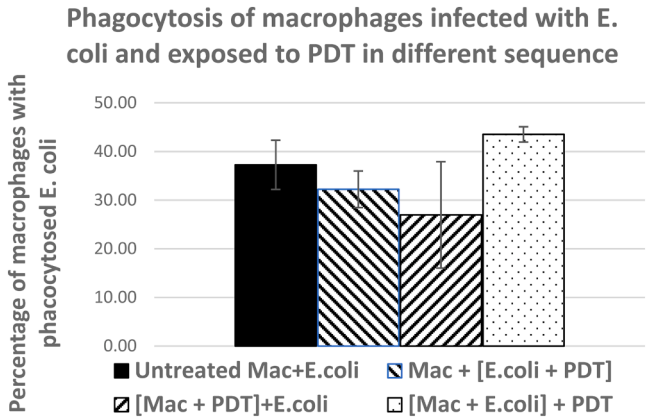

Fig. 4. Tracking of macrophage phagocytosis of FITC- labeled E. coli with or without PDT.

3.2. Adding melanoma

Following these investigations, we expanded the system to include a cancer cell (melanoma B16-F10). We documented its viability and toxicity using the selected PDT settings thus far and adhering to the same line of findings. Starting with melanoma alone, the results are presented in Fig. 5.

We observed that when melanoma is cultured alone, the PDT with parameters used in this experiment had no effect on it. Although PDT does not promote a major cell death from the exposure, there is a variation in toxicity, which is to be expected given the way PDT operates. The viability test shows no effect, despite a slight increase in toxicity of around 40 % following PDT. This indicates that the range of PDT dosages we utilized is appropriate for the investigations we had in mind when PDT has had some negative effects, but not enough big to alter viability.

3.3. Melanoma and macrophages

The next step of our investigation was testing PDT induced changes on the coculture of melanoma and macrophages, using the same logic as

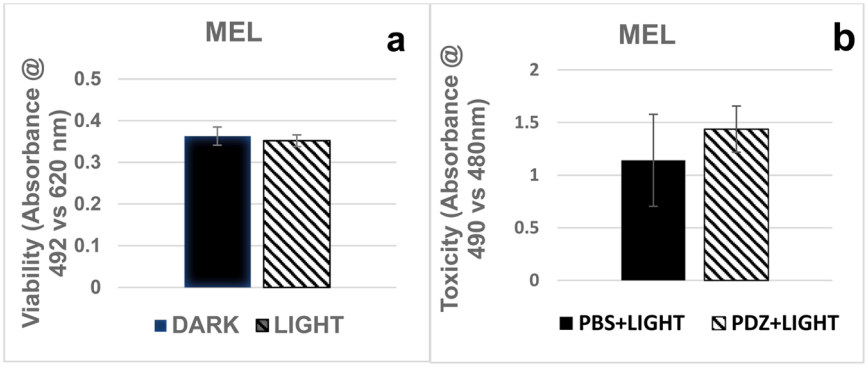

Fig. 5. (a)Viability of melanoma B16 with 1×10^{-4}mg/ml PDZ in the dark and after exposure to 30J/cm^2 of light at 660 nm (MTS assay). (b) Toxicity of melanoma B16 with 1×10^{-4}mg/ml PDZ in the dark and after exposure to 30J/cm^2 of light at 660 nm (LDH assay).

before. Fig. 6 displays the cell coculture viability and toxicity outcomes with and without PDT. The presence of macrophages in coculture appears to alter melanoma viability, reducing it by 22 % following PDT exposure. The reason for this could be that with the small, introduced toxicity in the melanoma by photodynamic effect, the macrophages action intensifies. PDT in the coculture has a similar effect on toxicity, increasing it by 28 %.

3.4. Melanoma + E. coli

Similarly, *E. coli* and melanoma were studied, with the findings displayed in Fig. 7. The presence of E. coli strongly affects the effect of PDT on the melanoma viability. The bacteria have a high photosensitizer uptake [17,18] and under oxidation can strongly modify the microenvironment of the cells. This has an indirect effect on melanoma, with considerable decrease of viability.

3.5. Melanoma + macrophages + E. coli

At last, all the elements were placed together, corresponding to more complexity. The context of an *in vitro* experiment, the presence of a cancer cell, an element of the immune system (macrophage), and an external infectant (*E. coli*) forms together the fundamental complex with functional and behavioral variability.

The results of viability and toxicity are presented in Fig. 8.

The decline in melanoma viability is clearly caused by the presence of macrophages. As discussed above, macrophages have the potential to amplify their effect under PDT. Additionally, because macrophages are close to melanoma and phagocytose highly sensitized [21] *E. coli*, they can further boost the production of reactive oxygen species (ROS), which damage surrounding cellular membranes of both macrophages and melanoma, causing as an overall effect severe decrease of viability

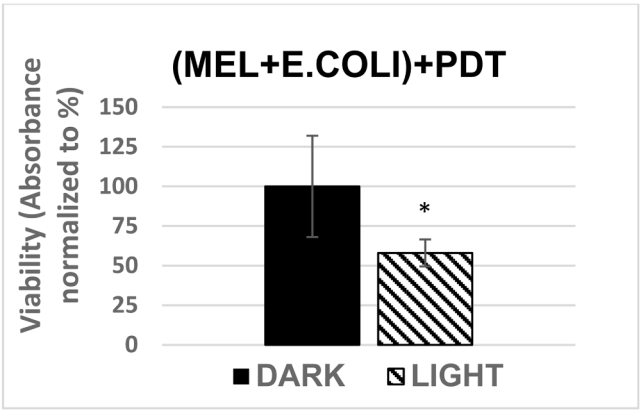

Fig. 7. Viability of melanoma B16 after incubation with 1×10^{-4}mg/ml Photodithiazide in the dark at 37C and infection with E. coli, then exposed to 30J/cm^2 of light at 660 nm (MTS assay). Data were analyzed by Student *t*-test and symbol * indicates p-value <0.05.

and increase of toxicity.

The toxicity testing validates the coordinated activity that *E. coli* and macrophages produce in melanoma. Toxicity rises by about 60 %, demonstrating the systemic nature of effects present in our system and the extent of bacterial effect. A variety of factors that make up the coculture are influenced by one another, adding processes that are absent when they are investigated separately or under restricted conditions. It is evident from the combined action of the three elements that the synergy of cells with varying functions and an outside agent can significantly alter the overall picture in a favorable way. These findings may lead to the development of numerous hypotheses. The presence of

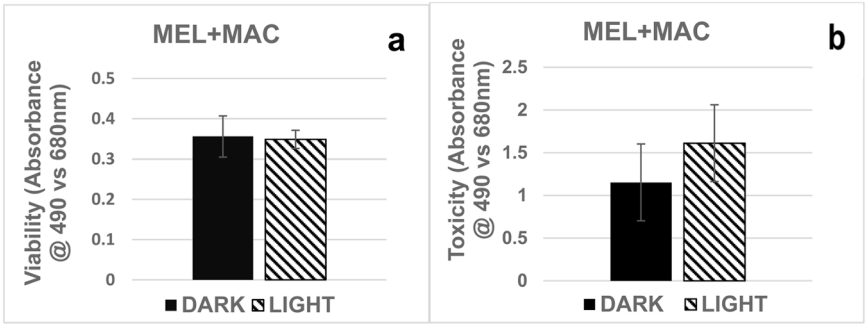

Fig. 6. (a) Viability of macrophages J771 coculture with melanoma B16 after incubation with 1×10^{-4}mg/ml of Photodithiazine in the dark at 37°C and after exposure to 30J/cm^2 of light at 660 nm (MTS assay). (b) Toxicity of macrophages J771 coculture with melanoma B16 after incubation with 1×10^{-4} mg/ml of Photodithiazine in the dark at 37°C and after exposure to 30J/cm^2 of light at 660 nm (LDH assay).

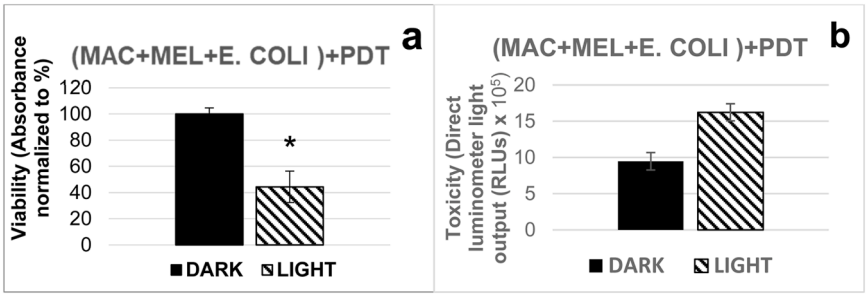

Fig. 8. (a)Viability of melanoma cells on the coculture of melanoma B16 grown with macrophages J771 after incubation with 1×10–04mg/ml of Photodithiazinein the dark at 37°C, infection with E. coli followed by exposure to 30J/cm^2 of light at 660 nm (MTS assay). (b) Toxicity of the melanoma cells on the coculture of melanoma B16 grown with macrophages J771 after incubation with 1×10^{-4}mg/mlof Photodithiazine e in the dark at 37 C, infection with *E. coli* and followed by exposure to 30J/cm^2 of light at 660 nm (Toxilight assay. Data were analyzed by Student *t*-test and symbol * indicates p-value <0.05.

the microbe may activate macrophages while also reducing the melanoma cell's resistance to macrophage assault. In this situation, the cancer cell's fragility intensifies the actions and ultimately leads to a better PDT response.

Our *in vitro* model is significant because it demonstrates the fundamental idea behind the tumor treatment when compared with the simplicity of the tumor cells alone. The *in vivo* microenvironment is made up of all kinds of cells and even infections, quite common in tumors and these there seem to be more than killing cells by ROS during PDT.

The microenvironment is crucial to the outcome in this context. In the current studies, we showed that the combination of an external infection (*E. coli*) and an immune system cell (macrophage) affects the subsequent PDT-related output in melanoma cells. Such circumstances should be considered when developing a detailed protocol for cancer therapy or even applying PDT in melanoma

Macrophages can be involved in improving or not outcome of cancer therapy. The immunological system in general is an important factor in any therapy. Photodynamic action can change the conditions and improve the overall results in treating tumors when the immune system cells are present. The presented results certainly indicate that PDT in the presence of macrophages promote more successful eradication of cancer cells. *In vitro* research of multicomponent elements may well be a way to understand basic observed features in PDT practice *in vivo* and shall promote new approaches for the therapeutics of PDT.

4. Conclusions

In this work we studied how introduction of *E. coli* and inducing the photodynamic effect influences immune response in cancer. We created a simple 2D *in vitro* "tumor model" of coculture of melanoma B16-F-10 with macrophages J774A.1 and infected it with *E. coli*. The cells responded differently individually, when compared to their behavior together. This is a characteristic very specific to each photosensitizer [19]. The influence of infection *E. coli* in melanoma resulted in significant loss of melanoma viability before and after applying PDT. This statistically significant effect was achieved not only because of *E. coli* bystander effect (reported earlier [20] expression of purine nucleoside phosphorylase (PNP), activating prodrugs toxic for bystander mammalian cells), but also because *E. coli* accumulates very strongly cationic PS used here [17] and produces strong phototoxicity in the cells as secondary effect.

The whole combination shows favorable elimination of melanoma cells by PDT. While this is still a demonstration of principles, the next stage would be an experiment to understanding the events recounted here to *in vivo* model, aiding in the development of more efficient methods to use PDT for melanoma.

CRediT authorship contribution statement

Barbara M. Kukiełczak Detweiler: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Amanda C. Zangirolami:** Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gus A. Wright:** Writing – review & editing, Methodology, Data curation. **Da M. Kim:** Methodology, Data curation. **Vanderlei S. Bagnato:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.pdpdt.2025.104518.

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